Discrimination of Methicillin-Resistant *Staphylococcus aureus* from Methicillin-Susceptible *Staphylococcus aureus* or Coagulase-Negative Staphylococci by Detection of Penicillin-Binding Protein 2 and Penicillin-Binding Protein 2' Using A Bioluminescent Enzyme Immunoassay

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Short Running Title

“Discrimination of MRSA from MSSA or CNS”

ABSTRACT

For the discrimination of methicillin-resistant *S. aureus* (MRSA) from methicillin-susceptible *S. aureus* (MSSA) or coagulase-negative staphylococci (CNS), we developed a bioluminescent enzyme immunoassay (BLEIA) for detecting penicillin-binding protein 2 (PBP2) and penicillin-binding protein 2' (PBP2') using biotinylated firefly luciferase. The BLEIA was able to detect recombinant PBP2 at 50 pg/ml and recombinant PBP2' at 500 pg/ml. PBP2 and PBP2' present in the membranes of *S. aureus* were extracted by acid and detergent treatment. The method was able to detect PBP2 or PBP2' extracted from 10^6 colony forming units of *S. aureus* because of efficient extraction and the high sensitivity of luciferase. In a study of clinical isolates previously characterized as either MRSA or MSSA by antibiotic susceptibility testing, all 34 specimens identified as MRSA were both PBP2 and PBP2' positive. The 34 MSSA specimens were PBP2 positive and PBP2' negative. Moreover, the BLEIA could detect PBP2' extracted from four species of methicillin-resistant CNS, but not PBP2 extracted from four species of methicillin-resistant and methicillin-susceptible CNS. This result suggested that PBP2 could be a unique marker for discrimination of *S. aureus* from CNS. A BLEIA that is able to detect PBP2 and PBP2' may be useful in clinical diagnostics.

Key words: coagulase-negative staphylococci, MRSA, PBP2', PBP2

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) have become a major problem in hospitals, causing serious staphylococcal infections including sepsis and endocarditis. The mechanism of methicillin resistance is based on the presence of an additional penicillin-binding protein, penicillin-binding protein 2' (PBP2'), which has a reduced affinity for beta-lactam antibiotics (Ubukata et al., 1985; Utsui and Yokota, 1985). Accurate and rapid detection of methicillin resistance in *S. aureus* is important for initiation of appropriate antimicrobial therapy in infected patients. However, the methods for identifying MRSA, such as susceptibility testing, are not always reliable because the phenotypic expression of methicillin resistance is known to be heterogeneous, depending on various factors, such as incubation time, temperature, and NaCl concentration. Moreover, these tests are time-consuming, requiring an initial culture period of 18–24 h followed by an additional 18–24 h for antibiotic susceptibility testing (Petersson et al., 1996). Some strains with borderline or low levels of resistance have also been found to not produce PBP2' (Hiramatsu et al., 1992).

Although polymerase chain reaction (PCR) can detect the *mecA* gene encoding PBP2', the potential for cross contamination, long turnaround times, the expense and availability of specialized equipment and trained staff may deter some laboratories from using PCR in clinical diagnosis (Barski et al., 1996; Bignardi et al., 1996; Murakami et al., 1991). Furthermore, some strains carrying the *mecA* gene may not exhibit methicillin resistance (Tokue et al., 1992).

A simple immunoassay method exists, which is based on a slide latex agglutination assay for directly detecting PBP2' from isolates of *S. aureus* (Nakatomi and Sugiyama, 1998). This method provides sensitive and specific immunodetection of MRSA in a pure culture by using anti-PBP2' antibodies, similar to the standard oxacillin disk diffusion or oxacillin salt agar screening (Arjanne et al., 1999). However, the specific immunodetection of MRSA based on PBP2' cannot be performed in the presence of methicillin-resistant *Staphylococcus epidermidis*, which is a frequent commensal contaminant of mixed flora samples (Cavassini et al., 1999). Indeed, the high level of sequence homology of the PBP2' present in *S. aureus*, *S. epidermidis*, and other potential coagulase-negative staphylococci (CNS) species (Wielders et al., 2001) precludes discrimination of methicillin-resistant strains of *S. aureus* from those of *S. epidermidis* or CNS (Hussain et al. 2000).

For discrimination of *S. aureus* from CNS, the coagulase testing is commonly used. But false results are sometimes given due to non-specifications, variability in plasma samples, and other staphylococci carrying coagulase (Mahoudeau et al., 1997; Sperber

and Tatani, 1975). A PCR assay using the *nuc* gene as a unique maker of *S. aureus* has been reported (Zhang et al., 2004). Considering the problems associated with detecting *mecA*, immunoassays are preferred.

In immunoassays, a unique maker of *S. aureus* has not been reported. Therefore we focused on PBP2, which is located in the cell wall and has a similar function to PBP2' (Pinho et al., 2001). Most Staphylococci contain PBP2 but the homology of PBP2 varies among staphylococci compared with PBP2' (Murakami et al., 1994), therefore there is some potential for using PBP2' to discriminate *S. aureus* from CNS.

For the detection of PBP2 and PBP2' by immunoassay, the extraction method of both antigens, which are located in the cell wall, is important because the antibody is inaccessible to antigens without some treatment. Furthermore, the method is required to be efficient, rapid and easy for use clinical laboratories. The extraction method for PBP2' using an alkali solution has been previously reported (Nakatomi and Sugiyama, 1998). However, there has been no study of the simultaneous extraction of PBP2 and PBP2'.

In this study, we had developed a bioluminescent enzyme immunoassay (BLEIA) using firefly luciferase for the detection of two antigens. Firefly luciferase emits luminescence (Ando et al., 2008) and a luciferase-luciferin reaction immediately generates stable bioluminescence. Moreover, because limited numbers of insects possess luciferase, the noise caused by accidental contamination is very low. Therefore, luciferase could have considerable advantages in terms of sensitivity and specificity compared with other enzyme labels in immunoassays. As a labeling enzyme, thermostable biotinylated firefly luciferase was developed by genetic engineering (Tatsumi et al., 1996). The thermostable biotinylated firefly luciferase was linked to streptavidin and a biotinylated antibody by an avidin-biotin complex in this BLEIA.

The BLEIA is able to detect PBP2 and PBP2' with high sensitivity and across a wide measurable range, and is suitable for the accurate diagnostics of MRSA. We examined the accuracy of the BLEIA by testing 68 clinical isolated strains of *S. aureus*. We also demonstrated the discrimination of MRSA from four species of CNS by detecting PBP2.

2. Materials and Methods

2.1. Bacterial strains

Tested bacteria were isolated from patients at Chiba University Hospital between 2009–2010. Species of all strains were identified with MicroScan WalkAway 96 and MicroScan Rapid Pos Combo panels (Siemens Healthcare Diagnostics, Inc). And

samples were tested for anti-microbial susceptibility by using a microbroth dilution method with a MicroScan WalkAway 96 and MicroScan Rapid Pos Combo panels. *S. aureus* with a minimum inhibitory concentration (MIC) > 4.0 µg/ml for oxacillin and CNS with a MIC > 0.25 µg/ml for oxacillin were defined as methicillin resistant. Bacteria were grown on a 5% sheep-blood agar prior to the BLEIA, PCR and MRSA screening test.

2.2. Production of recombinant PBP2 and PBP2'

The pET-16b vector (Invitrogen Corp.) was used to express recombinant PBP2 or PBP2' with a histidine (His) tag. The plasmids were transformed into *Escherichia coli* BL21 (DE3) and expression was induced by adding 1.0 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were disrupted using a French press and centrifuged at 9000 × g for 10 min. The supernatant was removed, and the cell pellet was suspended in 20 mM phosphate-buffered saline (PBS; pH 8.0), 130 mM NaCl, and 6.0 M urea. The soluble protein was loaded onto a HisTrap column (GE Healthcare) for purification, and elution was performed with 0.5 M imidazole. The concentration of the recombinant proteins was determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc.).

2.3. Production of monoclonal antibody

All monoclonal antibodies for PBP2 or PBP2' were kindly produced by Bio Matrix Research Inc. The immunogen of these antibodies was recombinant His tag-purified PBP2 or PBP2', as previously described (Kohler and Milstein, 1975).

2.4. BLEIA of PBP2, PBP2'

The biotinylated luciferase-streptavidin complex was prepared as previously described (Tatsumi et al., 1996; Fukuda et al., 2000). The BLEIA was based on a sandwich method. For coating, the capture antibody [50 µL of 4.0 µg/mL anti-PBP2 (clone 329) or anti-PBP2' (clone 682a)] was added to 96-well white microplates and incubated at room temperature for 2 h. The wells were washed three times with 300 µL of 60 mM Tris-HCl buffer (pH 8.0), 150 mM NaCl and 0.1% Tween 20 (TBS-T). After drying, 200 µL of TBS containing 3.0% bovine serum albumen (BSA) was added to each well for blocking, and incubated at room temperature for 1 h. After incubation, wells were washed three times with 300 µL of TBS-T. Then, 50 µL of samples were added to each well and incubated at room temperature for 1 h. The wells were washed three times with 300 µL of TBS-T and then 50 µL of 1.0 µg/mL biotinylated antibody against PBP2

(clone 446) or PBP2' (clone 560d) in TBS-T containing 0.5% BSA was added to each well and incubated at room temperature for 1 h. After incubation, the wells were washed three times with 300 μ L of TBS-T. In the next step, 50 μ L of 0.064 μ g/mL biotinylated luciferase-streptavidin complex was added to each well, and incubation was carried out for 30 min at room temperature. After the incubation, wells were washed six times with TBS-T, dried, and bioluminescent activities measured by adding 100 μ L substrate solution containing 0.94 mM luciferin, 2.0 mM ATP, 16 mM Mg_4CHCOO , and HEPES to each well. Luminescence intensity was measured with a XS3 LB960 microplate luminometer (Berthold Technol., Germany) integrating the emission for 20 s.

2.5. Extraction of PBP2' and PBP2

For efficient antigen extraction, five solutions were used. The extraction solutions were 0.1 M HCl, 2.0% AMPHITOL 20N (lauryl dimethylamine N- oxide, non-ionic detergent; KAO Co. Ltd, Japan), 0.1 M HCl containing 2.0% AMPHITOL 20N, 0.1 M HCl containing 2.0% Tween 20 and 0.1 M NaOH. MRSA or MSSA cultured on sheep blood agar plates were suspended in 200 μ L of each solution at a concentration of approximately 10^8 CFU/mL. The cell suspension was then boiled for 2 min and cooled to 4°C. The suspension containing 0.1 M HCl was added to 200 μ L of 0.1 M NaOH and 20 μ L of 0.4 M PBS (pH 8.0). The suspension containing 0.1 M NaOH was added to 200 μ L of 0.1 M HCl and 20 μ L of 0.4 M PBS (pH 8.0) for neutralization, followed by 200 μ L of H_2O and 20 μ L of 0.4 M PBS (pH 8.0). The suspensions were then centrifuged at $1,500 \times g$ for 5 min. Extracted proteins in the supernatant were examined by the developed BLEIA.

2.6. PCR detection of *mecA* and *pbp2*

Genomic DNA was extracted from MRSA or MSSA after a 30 min treatment at 37°C in TBS containing 100 mg of lysostaphin. Extracted DNA was purified by an ethanol precipitation method and the PCR was performed as previously described (24Coney et al., 1999). Thirty PCR cycles were used to amplify fragments with primers specific for *mecA* (5'-ATG CAG AAA GAC CAA AG-3' and 3'-ATA GAT GAA TAA TAA AAC A-5'). The primers used for *pbp2* detection were 5'-ATG ACG GAA AAC AAA GGA TC-3' and 5'-GGT GGA TTA ACA GGT ATA TTC AAC TAA-3'. Amplicons were separated by electrophoresis and detected under UV light.

2.7. Latex agglutination assays

Latex agglutination assays were performed using a MRSA screen test (Denka Seiken

Co.,Ltd. Japan) following the manufacturer's instructions.

3. Results

3.1. Construction and evaluation of BLEIA using recombinant PBP2, PBP2'

We developed a BLEIA for the detection PBP2 and PBP2'. The standard curves for recombinant PBP2 and PBP2' used in the BLEIA are shown in Figure 1. The detection limits ($0 \text{ pg/ml} \pm 3$ standard deviations) of PBP2 and PBP2' were 50 pg/ml and 500 pg/ml, respectively. Coefficients of variation (CV) for the range 50–500 ng/ml of PBP2 detection fell within the range of 0.7–11.4% and PBP2' fell within the range of 5.2–14.8%.

3.2. Extraction of PBP2 and PBP2' from *S. aureus*

We tested five extraction solutions to determine their efficiency and effectiveness for extracting PBP2 and PBP2' from MRSA isolates. The results of this comparison are shown in Figure 2. The concentrations of extracted antigen were measured by the BLEIA. The solution with highest luminescent intensity in the BLEIA was the 0.1 M HCl solution containing 2.0% AMPHITOL 20N for both PBP2 and PBP2' detection. The solution of 0.1 M HCl containing 2.0% AMPHITOL 20N was 500% more effective than the 0.1 M NaOH solution. For PBP2' extraction, the solutions without 0.1 M HCl containing 2.0% AMPHITOL were of approximately equal efficiency. The solutions containing 2.0% AMPHITOL 20N were more efficient than other solutions for PBP2 extraction regardless of whether HCl was present.

3.3. Detection of PBP2 and PBP2' extracted from *S. aureus* by BLEIA

We evaluated the assay range of the BLEIA for detecting both antigens extracted from *S. aureus*. Figure 3a shows the detection curve of PBP2 from MRSA or MSSA (range 1.0×10^5 to 1.0×10^9 CFU/ml). Antigens were extracted with 0.1 M HCl containing 2.0% AMPHITOL 20N. The detection limit was 0.5×10^6 CFU/ml for both MRSA and MSSA. Figure 3b shows the detection curve of PBP2' from MRSA or MSSA (range 1.0×10^5 to 1.0×10^9 CFU/ml). The detection limit was 0.5×10^6 CFU/ml for MRSA and PBP2' was not detected from MSSA.

3.4. Evaluation of the BLEIA on clinical samples

Thirty-four clinical samples of *S. aureus* identified as either MRSA or MSSA using a microbroth dilution method were tested by the BLEIA for PBP2' and PBP2. For assessment, it was decided that luminescence higher than the noise + 3 SD was a positive, anything below this would be judged as negative. The sample suspension was

prepared from a single colony of the isolation culture media. As shown in Table 1, PBP2 was detected in all samples of MRSA and MSSA and PBP2' was detected in all MRSA samples. PBP2' was not detected in any of the MSSA samples. These results corresponded to the PCR test results and those obtained from the MRSA screen test.

3.5. *PBP2 and PBP2' detection of CNS*

We evaluated whether it was possible to discriminate MRSA from CNS using the BLEIA; the results of this evaluation can be seen in Table 2. PBP2' was detected in each species of methicillin-resistant CNS but not in species of methicillin-sensitive CNS. PBP2 was not detected in four species; these results show that the presence of *S. aureus* was confirmed when PBP2 was detected in samples. When PBP2' only was detected, the sample was designated as methicillin-resistant CNS.

4. Discussion

In this study, we developed a BLEIA for the detection of PBP2 and PBP2' employing luciferase. The detection of PBP2 is a new approach for the discrimination of MRSA from CNS. The limits of detection were 50 pg/ml for PBP2 and 500 pg/ml for PBP2'. The differences in the limits of detection can be attributed to the affinity of antibody. Thus, we can conclude that the BLEIA using luciferase was highly sensitive and had a wide measurement range.

The antibody is inaccessible to PBP2 or PBP2' because both antigens are located in the cell membranes of *S. aureus*. Therefore, a simple and efficient extraction method is needed to facilitate detection of PBP2 and PBP2'. Additionally, it is necessary that the solution used for antigen extraction does not interfere with the antigen-antibody reaction. For example, Nakatomi and Sugiyama (1998) developed a simple method using a 0.1M NaOH solution in conjunction with sample boiling. This method does not interfere with the antigen-antibody reaction but the antigens were degraded by alkali treatment and strong boiling (data not shown). In such cases, the detection sensitivity of the BLEIA for PBP2 and PBP2' becomes low.

For the detection of the antigens, we developed a gentler method for antigen extraction using acid and detergents. We selected the non ionic detergent AMPHITOL20N from some commercially available detergents, because its ability to aid in antigen extraction was superior according to initial investigations (data not shown). Actually, the solution containing 0.1 M HCl and 2.0 % AMPHITOL20N was about 500 % more effective than an alkali solution for PBP2 or PBP2' extraction (Figure 2). Moreover, the detergent did not interfere with the antigen-antibody reaction (data not shown). A highly sensitive immunoassay is possible using the extraction solution we developed.

The BLEIA and an efficient extraction method have made possible highly sensitive detection of PBP2 or PBP2' from *S. aureus*. For accurate diagnosis of MRSA, this method requires more than 0.5×10^6 CFU/ml. Because the amount of bacterial in a single loopful cells or a single colony is more than 1.0×10^8 CFU/ml, the limit of detection of this method is sufficient to detect and discriminate between MRSA and MSSA from isolated strains. We have shown that 68 clinical isolates previously characterized as either MRSA or MSSA by susceptibility tests could be correctly identified from a single loopful of cells taken from sheep blood agar plates. PCR can detect the *mecA* gene with a limit of detection around 10^5 CFU/ml. However, some strains carrying the *mecA* gene are susceptible to methicillin. This suggests that the immunological detection of PBP2' is more appropriate.

PBP2' was detected in all methicillin-resistant strains of staphylococci (Table 2). This

result suggest that the detection of PBP2' only is not sufficient to strictly discriminate MRSA from other methicillin-resistant staphylococci. In our results, the developed BLEIA detected PBP2 in *S. aureus* only. The amino acid sequence homology of PBP2 is 87% among *S. aureus* and *S. warnri*, the highest homology from a BLAST search, although the homology of PBP2' is greater than 95% among staphylococci. Antibodies that are highly specific can generally distinguish such differences in homology. PBP2 was detected from only *S. aureus* meaning that the detection of PBP2 strictly discriminates *S. aureus* from other staphylococci. Additionally, the extracted samples contain both PBP2 and PBP2' because both antigens are located in the cell membrane. It is an advantage to use the detection of PBP2 to discriminate MRSA from CNS, because the extraction of samples does not need to occur separately.

In this study, we demonstrated the discrimination of MRSA from MSSA and CNS by a BLEIA that can detect PBP2 and PBP2'. As shown in Table 3, a PBP2' and PBP2 positive results is indicative of MRSA, a PBP2-positive only indicates MSSA, and a PBP2'-positive only tells us that an isolate is a methicillin-resistant CNS. When the BLEIA result is negative for both of PBP2 and PBP2', this suggests that the sample is a methicillin-susceptible CNS or another bacterium. Thus, the strict discrimination of MRSA from MSSA and CNS would be an important advantage in screening for MRSA or institution of appropriate anti-microbial therapy for patients in a clinical setting.

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REFERENCES

- Ando, Y., Niwa, K., Yamada, N., Nomoto, E. T., Irie, T., Kubota, H., Ohmiya, H., Akiyama, H., 2008. Firefly bioluminescence quantum yield and colour change by pH-sensitive green emission. *Nature Photon* 2, 44-47.
- Arjanne, G., Miranda, P., Nan, L., Max, H., Piet, W., Anton, B., Jan, K., 1999., Rapid Slide Latex Agglutination Test for Detection of Methicillin Resistance in *Staphylococcus aureus*. *J. Clin. Microbiol.* 39, 2789-2792.
- Barski P., Piechowicz L., Galinski J., and Kur J., 1996. Rapid assay for detection of methicillin-resistant *Staphylococcus aureus* using multiplex PCR. *Mol. Cell. Probes.* 10, 471-475.
- Bignardi, G. E., Woodford, N., Chapman, A., Johnson, A. P., and Speller, D. C. 1996., Detection of the *mec-A* gene and phenotypic detection of resistance in *Staphylococcus aureus* isolates with borderline or low-level methicillin resistance. *J. Antimicrob. Chemother.* 37, 53-63.
- Cavassini, M., Wenger, A., Jaton, K., Blanc, D. S., and Bille, J., 1999. Evaluation of MRSA-Screen, a simple anti-PBP 2a slide latex agglutination kit, for rapid detection of methicillin resistance in *Staphylococcus aureus*. *J. Clin. Microbiol.* 37, 1591-1594.
- Fukuda, S., Tatsumi, S., Igarashi, H., and Igimi, S., 2000. Rapid detection of *Staphylococcus aureus* using bioluminescent enzyme immunoassay. *Lett. App. Mico.* 31, 134-138.
- Hiramatsu, K., Kihara, H., and Yokota, T., 1992. Analysis of borderline-resistant *Staphylococcus aureus* using polymerase chain reaction. *Microbiol. Immunol.* 36, 445-453.
- Hussain, Z., Stoakes, L., Garrow, S., Longo, S., Fitzgerald, V., and Lannigan, R., 2000. Rapid detection of *mecA*-positive and *mecA*-negative coagulase negative staphylococci by an anti-penicillin binding protein 2a slide latex agglutination test. *J. Clin. Microbiol.* 38, 2051-2054.
- Kohler, G., and Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature.* 256, 495-497.
- Mahoudeau, I., Delabranche, X., Prevost, G., Monteil, H., and Piemont, Y., 1997. Frequency of isolation of *Staphylococcus intermedius* from humans. *J. Clin. Microbiol.* 35, 2153-2154.
- Murakami, K., Minamide, W., Wada, K., Nakamura, E., Teraoka, H., and Watanabe, S., 1991. Identification of methicillin-resistant strains of staphylococci by polymerase

- chain reaction. J. Clin. Microbiol. 29, 2240-2244.
- Murakami, K., Fujimura, T., and Doi, M., 1994. Nucleotide sequence of the structural gene for the penicillin-binding protein 2 of *Staphylococcus aureus* and the presence of a homologous gene in other staphylococci. FEMS. Microbiol. Lett. 117, 131-136.
- Nakatomi, Y., and Sugiyama, J. 1998. A rapid latex agglutination assay for the detection of penicillin-binding protein 2. Microbiol. Immunol. 42, 739-743.
- Petersson, A. C., Miorner, H., and Kamme, C., 1996. Identification of mecA related oxacillin resistance in staphylococci by the E test and the broth microdilution method. J. Antimicrob. Chemother. 37, 445-456.
- Pinho, M., G., Filipe, S., R., Lencastre, D., H., and Tomasz, A., 2001. Complementation of the essential peptidoglycan transpeptidase function of penicillin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in *Staphylococcus aureus*. J. Bacteriol. 183, 6525-6531.
- Sperber, W., H., and Tatani, S., R., 1975. Interpretation of the tube coagulase test for identification of *Staphylococcus aureus*. Appl. Microbiol. 29, 502-505.
- Tatsumi, H., Fukuda, S., Kikuchi, M., and Koyama, Y., 1996. Construction of biotinylated firefly luciferase using biotin acceptor peptides. Analytical. Biochemistry. 243, 176-180.
- Tokue, Y., Shoji, S., Satoh, K., Watanabe, A., and Motomiya, M., 1992. Comparison of a polymerase chain reaction assay and a conventional microbiologic method for detection of methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents. Chemother. 36, 6-9.
- Ubukata K., amashita N., and Konno M., 1985. Occurrence of a beta-lactam-inducible penicillin-binding protein in methicillin-resistant staphylococci. Antimicrob. Agents. Chemother. 27, 851-857.
- Utsui, Y., and Yokota, T., 1985. Role of an altered penicillin-binding proteins in methicillin- and cephem-resistant *Staphylococcus aureus*. Antimicrob. Agents. Chemother. 28, 397-403.
- Wielders, C., L., Vriens, M., R., Brisse, S., Graaf-Miltenburg, L., A., Troelstra, A., Fleer, A., Schmitz, F., J., Verhoef, J., and Fluit, A., C., 2001, Evidence for in-vivo transfer of mecA DNA between strains of *Staphylococcus aureus*. Lancet. 357, 1674-1675.
- Zhang, K., Sparling, J., Chow, B., L., Elsayed, S., Hussain, Z., Church, D., L., Gregson, D., B., Louie, T., and Conly, J., M., 2004. New quadriplex PCR assay for detection of methicillin and mupirocin resistance and simultaneous discrimination of *Staphylococcus aureus* from coagulase-negative staphylococci. J. Clin. Microbiol.

42, 4947-4955.

FIGURE CAPTIONS

Figure 1. Standard curves for recombinant PBP2 ($\square$) and recombinant PBP2' ($\bullet$) in the BLEIA. Results are the mean values ($n = 4$).

Figure 2. The extraction efficiency of PBP2 (Fig. 2a) and PBP2' (Fig. 2b) from MRSA with various solutions. PBP2 and PBP2' were detected by the BLEIA. The extraction was from 10^8 CFU of the same strain of bacteria. The boiling time of all extractions was 2 min. Results are the mean value ($n = 3$).

Figure 3. Relationship between cell amount and luminescent intensity of BLEIA. (a) PBP2 detection from MSSA ($\bullet$) and MRSA ($\square$). (b) PBP2' detection from MSSA ($\bullet$) and MRSA ($\square$). The same strain of MRSA or MSSA was used in (a) and (b).

TABLES

Table 1. Evaluation of BLEIA, PCR and MRSA screening test on *S. aureus* strains identified as either MRSA or MSSA.

Strains	Number of strains tested ^a	BLEIA positive strains		PCR test positive strains		MRSA-screen test
		PBP2	PBP2'	<i>pbp2</i>	<i>mecA</i>	
MRSA	34	34	34	34	34	34
MSSA	34	34	0	34	0	0

^aThe sample suspension was prepared from a single colony of the isolation culture media.

Table 2. Detection of PBP2 and PBP2' by the BLEIA after extraction with 0.1 M HCl and 2.0% AMPHITOL 20N for various CNS.

Strain	Susceptibility	BLEIA	
		PBP2	PBP2'
<i>S. caprae</i>	Resistant	-	+
<i>S. epidermidis</i>	Susceptible	-	-
<i>S. epidermidis</i>	Resistant	-	+
<i>S. haemolyticus</i>	Susceptible	-	-
<i>S. haemolyticus</i>	Resistant	-	+
<i>S. hominis</i>	Susceptible	-	-
<i>S. hominis</i>	Resistant	-	+
<i>S. warneri</i>	Susceptible	-	-
<i>S. warneri</i>	Resistant	-	+

Table3. Summary of the discrimination method using the detection result of PBP2 and PBP2'.

	PBP2' positive	PBP2' negative
PBP2 positive	MRSA	MSSA
PBP2 negative	Methicillin-resistant CNS	Methicillin-susceptible CNS or other bacterias

Figure 1.

Figure 2.

(a)

(b)

Figure 3.

(a)

(b)

List of Abbreviations

BLEIA, bioluminescent enzyme immunoassay; CFU, colony forming units; CNS, coagulase-negative staphylococci; CV, Coefficients of variation; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; PBP2, penicillin-binding protein 2; PBP2', penicillin-binding protein 2'; PCR, polymerase chain reaction; S, *Staphylococcus*; TBS, Tris-HCl buffer